

# Protein microarrays guide tolerizing DNA vaccine treatment of autoimmune encephalomyelitis

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The diversity of autoimmune responses poses a formidable challenge to the development of antigen-specific tolerizing therapy. We developed 'myelin proteome' microarrays to profile the evolution of autoantibody responses in experimental autoimmune encephalomyelitis (EAE), a model for multiple sclerosis (MS). Increased diversity of autoantibody responses in acute EAE predicted a more severe clinical course. Chronic EAE was associated with previously undescribed extensive intra- and intermolecular epitope spreading of autoreactive B-cell responses. Array analysis of autoantigens targeted in acute EAE was used to guide the choice of autoantigen cDNAs to be incorporated into expression plasmids so as to generate tolerizing vaccines. Tolerizing DNA vaccines encoding a greater number of array-determined myelin targets proved superior in treating established EAE and reduced epitope spreading of autoreactive B-cell responses. Proteomic monitoring of autoantibody responses provides a useful approach to monitor autoimmune disease and to develop and tailor disease- and patient-specific tolerizing DNA vaccines.

'Epitope spreading' probably evolved as a mechanism to prevent microbial mutagenic escape from host immune responses<sup>1</sup>, and in autoimmunity this normally protective mechanism goes awry. Epitope spreading defines the expansion of antigen-specific immune responses beyond those targeted in the initial immunization. When the new immune responses broaden to include additional determinants on the same protein, this is termed 'intramolecular epitope spreading', and when epitopes on different proteins are targeted, this is termed 'intermolecular epitope spreading'<sup>2,3</sup>. There is vigorous debate about whether epitope spreading is an essential step in the initiation and perpetuation of autoimmune disease or occurs as a consequence of local tissue damage.

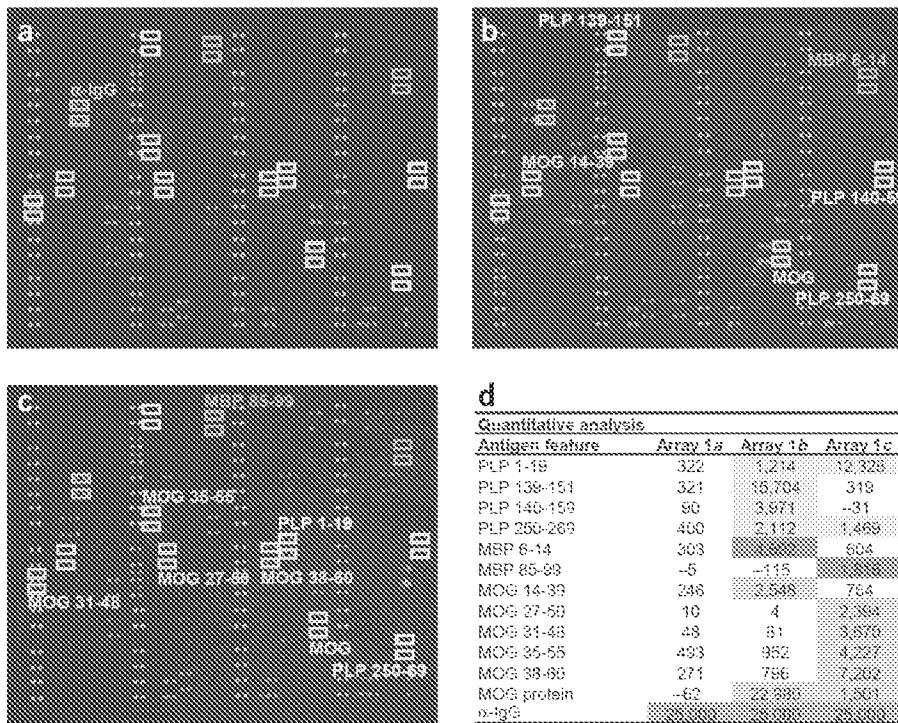
Human and murine systemic lupus erythematosus<sup>4</sup> and autoimmune diabetes<sup>5</sup> are associated with intra- and intermolecular spreading of autoreactive B-cell responses. EAE is an animal model for MS, a chronic inflammatory demyelinating disease of the central nervous system. EAE and MS are T-cell mediated, and in both diseases epitope spreading of autoreactive T-cell responses correlates with disease initiation and progression<sup>2,3,6</sup>. Autoantibodies targeting myelin oligodendrocyte glycoprotein (MOG) are probably pathogenic in EAE and

MS<sup>7,8</sup>, and autoantibodies recognizing other myelin proteins have been detected<sup>9,10</sup>. Nevertheless, the role of autoantibodies, B cells and epitope spreading in the pathogenesis of EAE and MS are poorly understood<sup>10,11</sup>. This is the first description of extensive epitope spreading of autoreactive B-cell responses in EAE.

The diversity of autoimmune responses poses great challenges to the development of antigen-specific tolerizing therapies, and new approaches are needed. Although DNA vaccines were initially used to stimulate immune responses against pathogens<sup>12,13</sup>, it was recently discovered that DNA vaccines encoding autoantigens induce specific immune tolerance<sup>10,14</sup>. Tolerizing DNA vaccines encoding myelin epitopes or proteins including proteolipid protein amino acids 139–151 (PLP(139–151)), myelin basic protein (MBP) or myelin oligodendrocyte glycoprotein (MOG) prevent induction of EAE<sup>10,14,15</sup>, and vaccines encoding glutamic acid decarboxylase or insulin prevent development of autoimmune diabetes in nonobese diabetic (NOD) mice<sup>16–18</sup>. DNA vaccines encoding autoantigen alone anergize autoreactive T cells<sup>14</sup>, whereas tolerizing vaccines incorporating interleukin-4 (IL-4) induce protective T-helper type 2 (T<sub>H</sub>2) responses<sup>10</sup>.

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**Figure 1** Myelin proteome arrays. Ordered antigen arrays were produced using a robotic microarrayer to attach myelin and control peptides and proteins, and antibodies to mouse IgG ( $\alpha$ -IgG), to poly-L-lysine-coated microscope slides. Arrays were probed with dilutions of mouse sera, and green features represent reactive antibodies detected using Cy3-conjugated anti-mouse IgM/G before scanning. The yellow features contain antibodies prelabeled with Cy3 and Cy5 to serve as 'reference' features to orient the arrays. (a-c) Individual arrays were probed with (a) normal control SJL mouse serum, which showed no autoantibody reactivity, and (b) serum from mice after a 10-week course of relapsing EAE induced with PLP(139-151) or (c) MBP(85-99). (d) Quantitative analysis of a-c with positive reactivities highlighted.

We describe an integrated approach to treat autoimmune disease using proteomic analysis of the specificity of autoantibody responses to guide development of genetic tolerizing vaccines. To survey autoantibody responses in EAE, we developed antigen microarrays<sup>19</sup> containing a spectrum of proteins and peptides derived from the myelin sheath, the target of the autoimmune response in EAE and MS<sup>9,20</sup>. We applied our myelin proteome arrays to study the evolution of the autoreactive B-cell responses in EAE, and to develop and monitor responses to DNA tolerizing vaccines.

## RESULTS

### Myelin proteome arrays

The 2,304-feature myelin proteome arrays contain  $\leq 232$  distinct antigens, including proteins and sets of overlapping peptides representing MBP, PLP, MOG, myelin-associated oligodendrocytic basic protein (MBOP), oligodendrocyte-specific protein (OSP),  $\alpha$ B-crystallin, cyclic nucleotide phosphodiesterase (CNPase) and Golli-MBP. We used myelin proteome arrays to profile autoantibody responses in serum derived from mice with EAE, and images of representative arrays are presented (Fig. 1). No autoantibody reactivity was detected in serum from a healthy SJL/J mouse (Fig. 1a,d). Arrays incubated with serum derived from two mice with relapsing EAE induced with PLP(139-151) (Fig. 1b) and MBP(85-99) (Fig. 1c) revealed that both mice developed autoantibody reactivity against epitopes derived from PLP, MBP and

MOG, but that there were differences in the fine specificity of their autoreactive B-cell responses. The first EAE mouse reacts with PLP(139-151), MBP(6-14) and MOG(14-39) (Fig. 1b,d), whereas the second differentially reacts with PLP(1-19), MBP(85-99), MOG(27-50), MOG(31-48), MOG(35-55) and MOG(38-60) (Fig. 1c,d).

### Array validation

Incubation of myelin proteome arrays with antibodies specific for PLP(139-151), PLP(178-191), MOG(35-55), MBP(68-86), MBP(82-87) and MBP(85-99) revealed specific detection of their corresponding reactivities (Fig. 2a). Comparison of array and enzyme-linked immunosorbent assay (ELISA) analysis of serum samples derived from EAE and control mice showed concordant results for autoantibody reactivity against PLP(139-151), PLP(178-191) and MOG(35-55) (Fig. 2b-d). We showed earlier that antigen arrays are four- to eightfold more sensitive than conventional ELISA, provide consistent intra- and interassay results, and detect autoantibodies in a linear fashion over a 3-log range down to nanogram-per-milliliter concentrations<sup>19</sup>.

### Autoantibody diversity predicts disease severity

To characterize autoreactive B-cell responses in acute EAE, we induced SJL/J mice to develop EAE with one of three different encephalitogenic myelin antigens: PLP(139-151), MBP(85-99) or spinal cord

homogenate (SCH). Approximately 7 d after onset and after partial recovery from acute EAE, we obtained serum and carried out myelin proteome array analysis. The significance analysis of microarrays (SAM) algorithm<sup>21</sup> was applied to identify antigen features with statistically significant differences in array reactivity between groups of mice induced for EAE with distinct encephalitogens. A hierarchical cluster algorithm using a pairwise similarity function<sup>22</sup> was then used to order mice and SAM-selected antigen features on the basis of the degree of similarity in their autoantibody reactivity profiles (Fig. 3a).

The capacity to use mathematical transformations to cluster groups of mice with similar patterns of reactivity to different antigenic epitopes allowed us to see patterns that were not obviously apparent. The specific antigen used for immunization induced different patterns in the autoantibody response. Mice induced for EAE with PLP(139-151) clustered and showed strong reactivity against PLP(139-151) as well as weak reactivity against MOG and several PLP and MBP peptides (Fig. 3a). Mice induced with MBP(85-99) also clustered, but in contrast demonstrated strong reactivity against MBP(85-99) and weak reactivity against several MBP- and PLP-derived peptides (Fig. 3a). SCH-induced mice clustered and showed weak reactivity against a variety of MBP, MOG and PLP epitopes (Fig. 3a). These genetically identical groups of mice induced for EAE with different encephalitogens showed reactivity against both shared and distinct sets of MBP, PLP and MOG peptides.

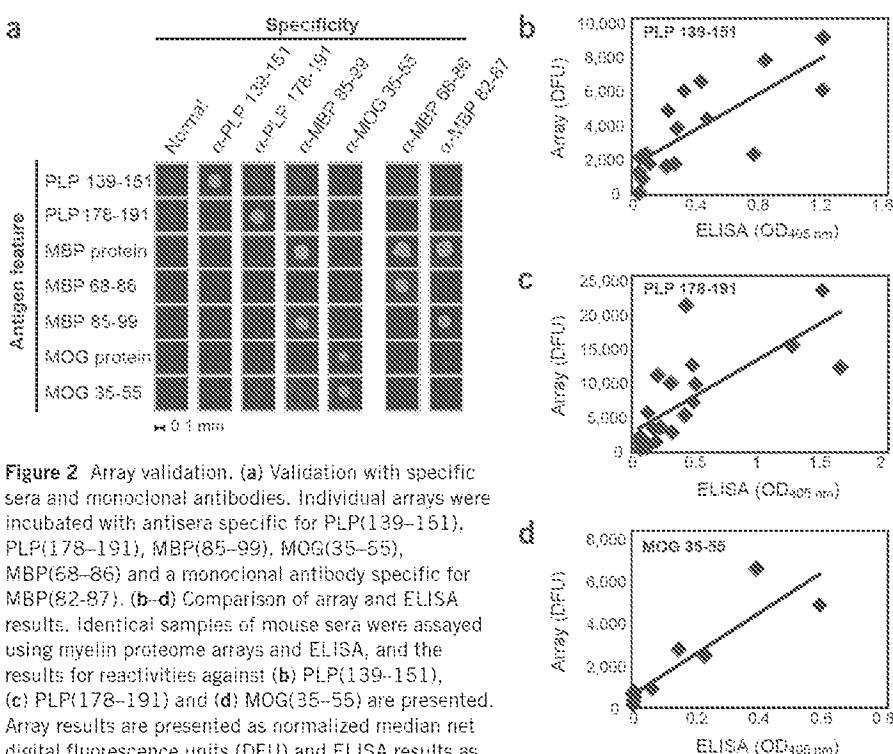
After partial recovery from acute EAE, within groups of mice induced with PLP(139–151) or MBP(85–99) we identified subclusters of mice on the basis of differences in the diversity of their autoantibody responses (Fig. 3a). Subclusters with increased diversity of autoantibody reactivity subsequently showed increased disease relapse rates. Characterization of the differences between array results from mice with the least and greatest number of relapses within each group showed increased reactivity against a spectrum of myelin epitopes in mice that subsequently developed more active disease (Fig. 3b,c). Thus, increased diversity of the autoreactive B-cell response in acute EAE predicted a more severe subsequent disease course.

#### Anti-myelin B-cell responses spread in relapsing EAE

Array analysis was done on paired samples obtained from these groups of mice induced with different encephalitogens after recovery from the acute episode of paralysis and after a 10-week course of relapsing EAE. SAM and hierarchical cluster analysis of array results showed that development of relapsing EAE was associated with extensive intra- and intermolecular spreading of autoantibody responses to overlapping but distinct sets of epitopes on myelin proteins including PLP, MBP, MOG and CNPase (Fig. 4). Groups of mice differentially targeted certain epitopes. For example, PLP(139–151)-induced mice clustered and differentially targeted MBP(71–89), whereas MBP(85–99)-induced mice clustered and targeted MBP(141–159). Furthermore, the inducing encephalitogenic peptide remained the dominant target of the autoreactive B-cell response (Fig. 4). There was heterogeneity in the fine specificity of the autoantibody responses between groups of mice induced with different encephalitogens, and between mice within each group (Figs. 3 and 4). All groups of mice with relapsing EAE targeted a set of common epitopes including PLP(50–69), MBP(131–153) and MOG(63–87).

#### Cocktail tolerizing DNA vaccine improves clinical outcome

On day 17 after recovery from acute EAE induced with PLP(139–151), myelin proteome array analysis revealed autoantibody reactivity directed against PLP, MBP and MOG (Fig. 3a). Although myelin-associated glycoprotein (MAG) epitopes were not included on the arrays described, in later experiments MAG peptides were added to the arrays and considerable autoantibody reactivity was observed in relation to several MAG epitopes (see Supplementary Fig. 1 online). On the basis of this proteomic profile, we generated tolerizing DNA vaccines encoding these array-determined targets. Full-length cDNAs encoding MBP, MOG, MAG and PLP were amplified from mouse brain cDNA using PCR and cloned into a mammalian expression vector. Relapsing EAE was induced in SJL/J mice by immunization with encephalitogenic PLP(139–151). Beginning on day 17 after recovery from the acute paralytic attack in EAE (7–8 d after disease onset), mice were injected intramuscularly on a weekly basis with control therapies or DNA encoding a cocktail of array-determined myelin targets, with or without DNA encoding the T<sub>H</sub>2-driving cytokine IL-4. In comparison with control therapies, the relapse rates



**Figure 2** Array validation. (a) Validation with specific sera and monoclonal antibodies. Individual arrays were incubated with antisera specific for PLP(139–151), PLP(178–191), MBP(85–99), MOG(35–55), MBP(68–86) and a monoclonal antibody specific for MBP(82–87). (b–d) Comparison of array and ELISA results. Identical samples of mouse sera were assayed using myelin proteome arrays and ELISA, and the results for reactivities against (b) PLP(139–151), (c) PLP(178–191) and (d) MOG(35–55) are presented. Array results are presented as normalized median net digital fluorescence units (DFU) and ELISA results as optical densities (OD).

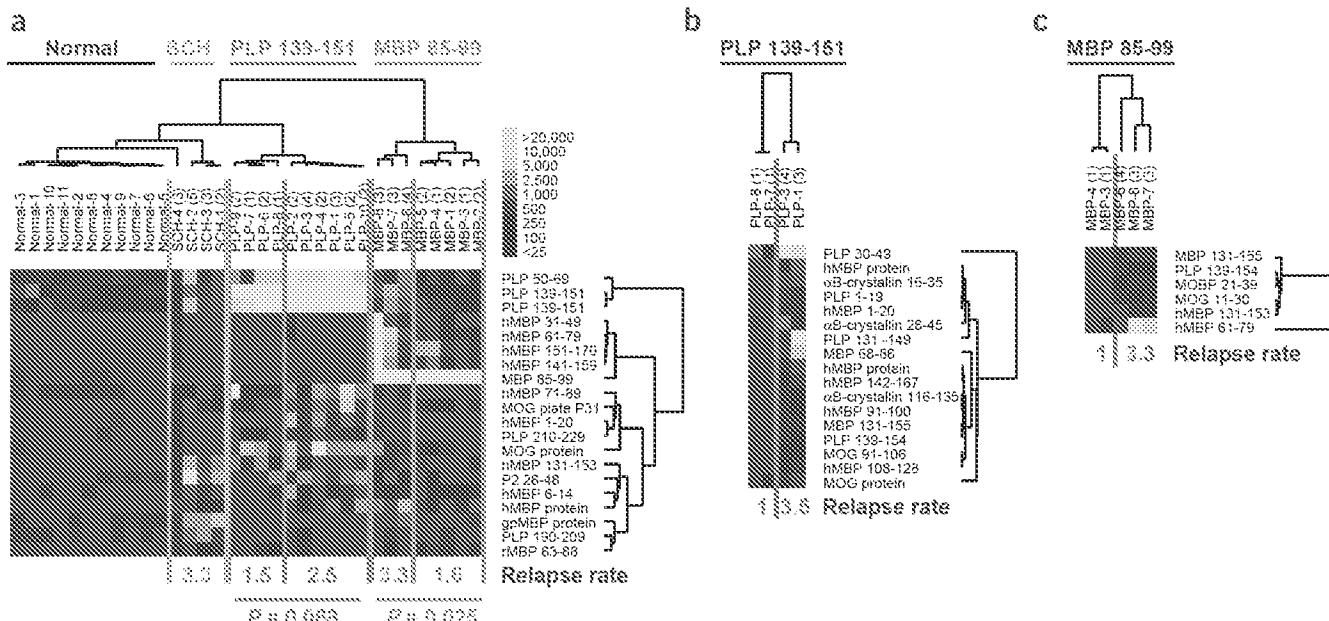
of mice treated with DNA cocktail alone or DNA cocktail plus IL-4 were lower by 42% ( $P = 0.026$ ) and 65% ( $P = 0.001$ ), respectively (Table 1). DNA cocktail plus IL-4 was more effective at reducing the relapse rate than DNA encoding the myelin epitope PLP(139–151) plus IL-4 ( $P = 0.006$ , by Mann-Whitney test).

#### Reduced epitope spreading in mice with improved outcomes

We conducted myelin proteome array analysis to determine if efficacious tolerizing DNA therapy altered the autoantibody profile. Array analysis was done on serum obtained from mice with relapsing EAE after a 10-week course of treatment. Mice treated with the efficacious DNA cocktail or DNA cocktail plus IL-4 cluster showed reduced epitope spreading of autoreactive B-cell responses (Fig. 5a). In contrast, mice receiving control therapies did not discretely cluster and underwent extensive spreading of their autoreactive B-cell responses to epitopes on myelin proteins including MBP, PLP and MOG. DNA encoding PLP(139–151) showed less efficacy in reducing EAE and was associated with a smaller reduction in epitope spreading (Fig. 5b). These results demonstrate that efficacious tolerizing therapy can reduce epitope spreading of autoreactive B-cell responses.

#### DISCUSSION

MS and EAE are characterized by clinical subtypes that include a relapsing-remitting pattern of disease activity, with periodic exacerbations of neurological dysfunction that frequently lead to accumulating disability. The most widely used drugs for the treatment of MS, type 1  $\beta$ -interferon preparations and glatiramer acetate, were approved for clinical use on the basis of reduction of relapse frequency<sup>23</sup>. It is hypothesized that epitope spreading may drive clinical relapses in EAE and MS<sup>3</sup>. We developed and applied myelin proteome microarrays to characterize the evolution of autoreactive B-cell responses in acute and chronic EAE, and in response to antigen-specific tolerizing therapy.



**Figure 3** The diversity of autoreactive antibody responses in acute EAE predicts subsequent disease activity. (a) Hierarchical clustering of antigen features with statistically significant differences in myelin proteome array reactivity between sera derived from groups of normal control mice and from groups of mice upon recovery from acute EAE induced with PLP(139–151) (day 17), MBP(85–99) (day 22) or SCH (day 25). Mice were later scored daily for 10 weeks to determine the number of relapses for each mouse (indicated in parentheses). The average relapse rates for mice included in the primary subnodes of the dendrogram, and *P* values by Mann-Whitney test for the differences in relapse rate between these nodes, are indicated. (b,c) Antigen features with statistically significant differences in array reactivity between subsets of mice with the greatest (three and four) and least (one) number of relapses within groups induced for EAE with PLP(139–151) or MBP(85–99). For Figures 3–5, SAM<sup>21</sup> was used to identify antigen features with statistically significant differences in array reactivity between groups of mice. A hierarchical cluster algorithm based on a pairwise similarity function<sup>22</sup> was applied to order mice based on similarities in their array reactivities for the SAM-identified features (dendograms depicting cluster relationships are displayed above the individual mice), and to order antigen features based on similarities in reactivities in the mice studied (dendograms displayed to the right). Relationships between mice or antigen features are represented by tree dendograms whose branch lengths reflect the degree of similarity in array reactivity determined by the hierarchical cluster algorithm. After clustering, labels were added above the dendograms to indicate the location of clusters of mice induced for EAE with different encephalitogens.

We showed that greater diversity of autoreactive B-cell responses at the time of recovery from acute EAE predicted increased subsequent disease activity (Fig. 3a–c) and that extensive intra- and intermolecular epitope spreading occurs in chronic EAE (Fig. 4). Although PLP, MBP, MOG and MAG had earlier been identified as potential targets of autoimmune responses in SJL EAE<sup>3,24</sup>, simultaneous targeting of these and other myelin proteins has not been shown before. Such extensive diversity and spreading of autoreactive B-cell responses suggests that autoimmune responses may be considerably broader than recognized in the past, and this has important implications for the design of antigen-specific tolerizing therapies. The high sensitivity of antigen arrays<sup>19</sup> facilitated early detection of diverse B-cell responses, and such increased, and possibly rapid, diversification is probably physiologically adaptive for combating microbes.

Our data show that the diversity of autoreactive B-cell responses in acute EAE predicts subsequent disease activity (Fig. 3), suggesting that diverse autoreactive B-cell responses precede and contribute to autoimmune disease progression<sup>25</sup>. SJL mice induced with the same encephalitogen possess autoantibody specificity profiles that include both dominant and scattered antigen feature reactivities, suggesting a component of random variation (Figs. 3a and 4). Mice with increased scattering (diversity) subsequently relapsed at higher rates (Fig. 3). Myelin array analysis of larger sample sets will be necessary to determine if reactivity to individual or combinations of specific epitopes, or increased diversity itself, is associated with development of more

severe EAE. We are now testing the hypothesis that stochastic variations in the selection and activation of lymphocyte repertoires predispose individuals to *de novo* development of autoimmunity, which is also more severe. This hypothesis could explain the paradox that substantial discordance exists in the incidence of autoimmunity in genetically identical humans and rodents<sup>9,26</sup>.

Epitope spreading of autoreactive B-cell responses is apparent much earlier (at day 17) than the described epitope spreading of autoreactive CD4<sup>+</sup> T-cell responses that occurs 3–8 weeks after disease induction<sup>2,3,6</sup>. The amplitude and kinetics of anti-myelin B-cell responses in acute EAE are consistent with activation of diverse initial B-cell responses, and not with classical secondary B-cell responses (Fig. 3a). Diverse anti-myelin B cells are probably activated through the destruction of myelin and activation of the inflammatory cascade by the initiating autoreactive T cells. The diverse autoreactive B-cell responses in acute EAE (Fig. 3a) could contribute to activation of T-cell responses to new myelin epitopes that together drive epitope spreading to immunodominant and cryptic myelin epitopes and thereby perpetuate autoimmunity (Fig. 4)<sup>25</sup>.

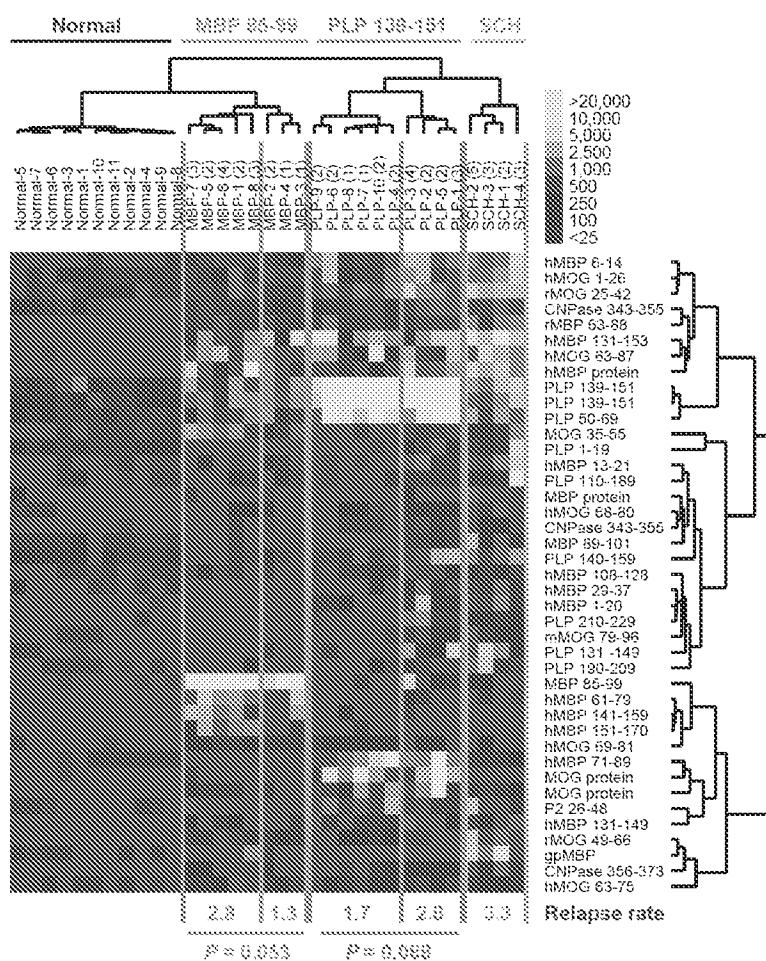
In infectious immunity, the original immunogen often remains paramount in B- and T-cell memory responses, a phenomenon termed ‘original antigenic sin’<sup>27,28</sup>, whereas in autoimmunity the initial T-cell response often disappears<sup>29,30</sup>. In contrast to the migratory T-cell reactivity in EAE<sup>29,30</sup>, we observed persistence of strong autoantibody reactivity against the inciting autoantigen (Fig. 4).

The diversity of autoimmune responses challenges the idea that simplistic protein- and epitope-based tolerizing therapies targeting a single epitope of one protein, or even a whole protein, could be efficacious<sup>31,32</sup>. We formulated the DNA treatment to encode array-determined targets including full-length PLP, MBP, MOG and MAG. This tolerizing DNA vaccine encoding a cocktail of myelin proteins treated established autoimmune responses targeting diverse myelin epitopes and proteins (Fig. 3a). We had earlier shown that tolerizing DNA vaccines encoding PLP(139–151) plus IL-4 induced protective T<sub>H</sub>2 responses<sup>10</sup>. Addition of DNA encoding the T<sub>H</sub>2-driving cytokine to the DNA cocktail showed a trend toward increased efficacy over the DNA cocktail alone (Table 1). In separate experiments the myelin cocktail tolerizing DNA vaccine, both with and without DNA encoding IL-4, prevented the induction of EAE (data not shown).

Myelin proteome array analysis showed broad reductions in autoreactive B-cell epitope spreading after treatment with the cocktail tolerizing DNA vaccine (Fig. 5), suggesting induction of tolerance to multiple epitopes on multiple proteins encoded in this tolerizing vaccine. Although others have reported that interventions preventing EAE concomitantly prevented epitope spreading<sup>33,34</sup>, our results show that proteomic monitoring of epitope spreading can be used to follow responses to tolerizing therapy in established autoimmunity.

Major obstacles to the development of antigen-specific therapies to treat autoimmune disease have included (i) a lack of methods to determine the specificity of autoimmune responses, for which proteomic analysis of autoantibody responses represents a useful tool, and (ii) a lack of methods to induce antigen-specific tolerance, for which genetic tolerizing vaccines are a promising approach. Our data show that treatment of mice with established EAE with tolerizing DNA vaccines encoding multiple array-determined myelin targets of the autoantibody response in acute EAE provided efficacious therapy (Figs. 3 and 5, Table 1). We have demonstrated treatment of autoimmune disease with tolerizing DNA vaccines encoding array-determined autoantigen targets. Our approach is also supported by the efficacy of tolerizing DNA vaccines encoding insulin and glutamic acid decarboxylase in treating prediabetic NOD mice<sup>16–18</sup> at an age when they show ongoing insulitis and serum autoantibodies against these self-antigens<sup>25,36</sup>.

Our approach is rooted in the hypothesis that concordance exists between the specificity of the autoreactivity B- and helper T-cell responses at the protein level<sup>37</sup>. Although examples exist of concordance and discordance in the fine specificity of autoreactive B- and T-cell responses<sup>38</sup>, the reciprocal nature of B-T activation drives concordance at the macromolecular level. We propose that the specificity of autoantibody responses reflects the overall specificity of autoimmune responses and can be used to identify autoantigens and to



**Figure 4** Extensive intra- and intermolecular spreading of autoreactive B-cell responses, with persistence of reactivity against the inducing encephalitogen, in chronic relapsing EAE. The mice described in **Figure 3a** were followed over a 10-week course of relapsing EAE, after which sera were collected and array analysis conducted. Hierarchical clustering was applied to order antigen features identified as having significant differences in array reactivity between the groups of mice. Relapse rates for individual mice are in parentheses and average relapse rates for dendrogram subnodes are indicated along the base.

select disease- and patient-specific tolerizing therapy. We show that tolerizing DNA vaccines encoding multiple targets identified with microarrays tolerized diverse autoimmune responses to treat established EAE, even when therapy began after an acute attack of paralysis (Table 1). The frequency of subsequent relapses was diminished.

The approach offers important advantages over genomics-based discovery strategies that require complex, time-consuming and expensive preclinical development. Identification of an autoantigen target with an array is followed by use of the PCR to rapidly clone the offending autoantigen into the tolerizing DNA vector. The rapidly devised 'drug' can then be evaluated in a relevant animal model of autoimmunity. Large-scale analysis of autoantibody responses can also be applied to develop and select other antigen-specific tolerizing therapies, including delivery of autoantigen-derived peptides, polypeptides and other biomolecules<sup>37</sup>.

Protein array monitoring of autoantibody responses has the potential to improve care for patients with autoimmune diseases by permitting identification of 'biosignatures' for diagnosis, prognostication and guidance of tolerizing therapy.

## METHODS

**Peptides, proteins and antibodies.** Myelin proteome arrays contained  $\leq 13$  proteins and 219 synthetic peptides, including 4 proteins and 85 peptides from MBP, 3 proteins and 30 peptides from PLP, 3 proteins and 50 peptides from MOG, 2 peptides from MBP, 1 protein and 16 peptides from  $\alpha$ B-crystallin, 20 peptides from CNPase, 1 protein and 11 peptides from peripheral myelin protein 2 (P2), 2 peptides from the acetylcholine receptor and 4 nonmyelin peptides or proteins (see Supplementary Methods online for a detailed list). Mouse antiserum specific for PLP(139–151), PLP(178–191), MBP(85–99) and MOG(35–55) were generated by immunizing SJL or C57BL/6 mice subcutaneously with 100  $\mu$ g of the relevant peptide emulsified in complete Freund's adjuvant (CFA), and serum was collected at day 13. Rat antiserum specific for MBP(68–86) was generated by immunizing Lewis rats with MBP(68–86) emulsified in CFA. The rat monoclonal antibody specific for MBP(82–87) was obtained from Serotec.

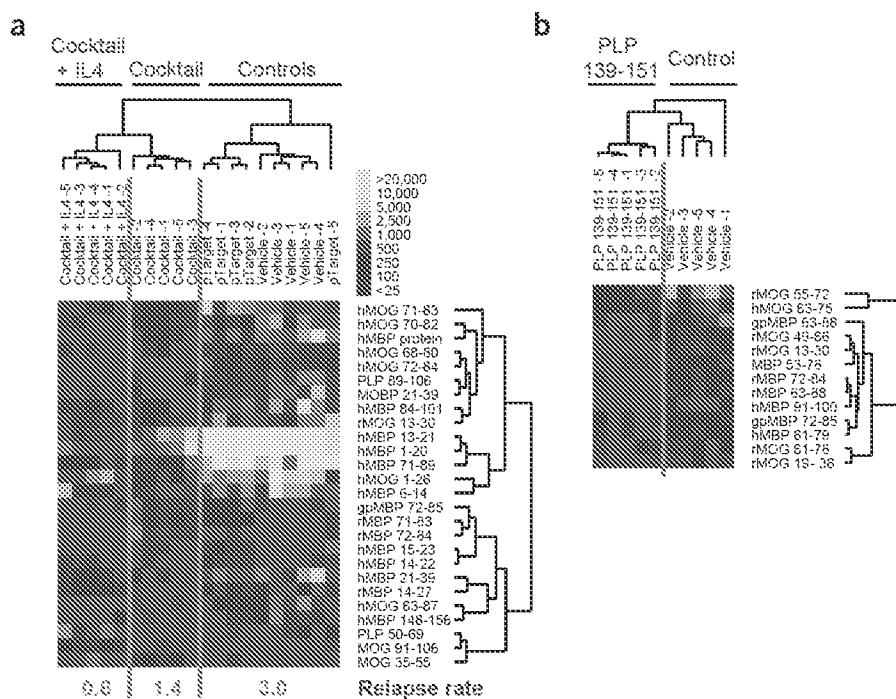
**Array production and probing.** Myelin proteome arrays were produced using a robotic arrayer to attach peptides and proteins to poly-L-lysine-coated slides (CEL Associates) in an ordered array<sup>19,39</sup>. On each array were printed 4–12 replicate features of each peptide or protein. Arrays were circumscribed with a hydrophobic marker, blocked overnight at 4 °C in PBS containing 3% FCS and 0.5% Tween-20, incubated with 1:150 dilutions of mouse serum in blocking buffer for 1 h at 4 °C, and washed twice for 20 min rotating in blocking buffer. Arrays were incubated with 1:4,000 dilutions of cyanin 3 dye (Cy3)-conjugated goat anti-mouse IgM/G or goat anti-rat IgM/G (Jackson ImmunoResearch) for 1 h at 4 °C and then washed twice for 30 min in blocking buffer, twice for 30 min in PBS and twice for 15 s in water. Arrays were spun dry and scanned with a GenePix 4000B array scanner (Axon Instruments). Detailed protocols are published<sup>19</sup> and are available online at <http://www.stanford.edu/group/antigenarrays/>. False-color images derived from the scanned digital images are presented.

**ELISA.** ELISA was conducted as described<sup>40</sup>. Antibody binding was detected using alkaline phosphatase-conjugated monoclonal goat anti-mouse IgG<sub>1</sub> or IgG<sub>2a</sub> (Southern Biotechnology Associates), and reported titers represent the sum of the IgG<sub>1</sub> and IgG<sub>2a</sub> results.

**Table 1** DNA constructs encoding array-identified targets in acute EAE treat established EAE<sup>a</sup>

DNA	n	Relapses	Mean relapse rate	Relapse rate P value compared to vehicle	No. with no relapses (%)	No. with no relapses P value compared to vehicle
Vehicle	20	52	2.6	—	1 (5%)	—
IL-4	14	44	3.1	0.419	0	1.00
PLP(139–151)	17	35	2.1	0.284	2 (12%)	0.584
+ IL-4						
Cocktail	18	27	1.5	0.026	4 (22%)	0.170
Cocktail + IL-4	17	16	0.9	0.001	7 (41%)	0.014

<sup>a</sup>Full-length cDNA encoding array-identified targets including MBP, PLP, MOG and MAG were amplified from mouse brain cDNA by PCR and cloned into the pTARGET mammalian expression vector. At 7 d after onset of and after partial recovery from acute paralytic EAE (day 17) induced with PLP(139–151), SJL/J mice were treated with DNA expressing (i) IL-4, (ii) PLP(139–151) and IL-4, (iii) MBP, PLP, MOG and MAG (cocktail), or (iv) cocktail and IL-4. P values provided for comparison of mean relapse rates by Mann-Whitney test, and number of mice with no relapses by Fisher's exact test. The experiment presented is representative of three independent experiments.



**Figure 5** Tolerizing DNA vaccines reduce autoantibody epitope spreading. (a) At day 7 after onset of and after partial recovery from acute paralytic EAE (day 17) induced with PLP(139–151), SJL/J mice were treated weekly with PBS vehicle, empty pTARGET vector, pTARGET expressing MBP, PLP, MOG and MAG (cocktail), or pTARGET expressing the cocktail and IL-4. After the 10-week treatment, serum was obtained, array analysis carried out and SAM used to identify and hierarchical cluster analysis to order antigen features. (b) Mice with established EAE were treated with PBS vehicle or pVAX vector encoding PLP(139–151) for 6 weeks, after which serum was obtained and array analysis conducted.

**Array data analysis.** GenePix Pro 3.0 software (Axon Instruments) was used to determine the net median pixel intensities for individual features. Normalized median net digital fluorescence units (DFUs) represent median values from 4–12 identical antigen features on each array normalized to the median intensity of 8 anti-IgG features, so that the normalized anti-IgG reactivity was 25,000 for all arrays. For Figure 1d, positive reactivity was set at fourfold above the median of four sets of negative control features. SAM<sup>21,41</sup> was applied to identify antigens with statistically significant differences in array reactivity between groups of EAE animals or EAE animals and controls. SAM ranks each antigen on the basis of a score obtained by dividing the differences between the mean reactivities for each group by a function of their standard deviations, and then estimates a false discovery rate (FDR) for each antigen by permuting the repeated measurements between groups. Log base 2 of adjusted array values (values <10 were set to 10 and resulting values divided by 300; antigen features with no variation between arrays were eliminated) were input into SAM and results selected on the basis of criteria that included FDR <0.05 (except for Figs. 3c and 5b, for which the FDR was <0.06) combined with numerator thresholds of 0.7572 (Figs. 3a and 4), 3.2 (Figs. 3b,c) or 1.42 (Fig. 5). SAM results were arranged into relationships using Cluster<sup>22</sup>, and for Figure 3a PLP(139–151) and MBP(85–99) were weighted. Cluster results were displayed using TreeView<sup>22</sup>.

**EAE.** EAE was induced in SJL/J mice by subcutaneous injection of PLP(139–151) (100  $\mu$ g/mouse), MBP(85–99) (200  $\mu$ g/mouse) or SCH (200  $\mu$ g/mouse) emulsified in CFA containing 4 mg/ml heat-killed *Mycobacterium tuberculosis*

H37Ra (Difco Laboratories). Mice induced with MBP(85–99) or SCH were injected intravenously on the day of immunization and 48 h later with 0.1 ml of 4 µg/ml *Bordetella pertussis* toxin. Mice were scored daily for EAE as described elsewhere<sup>10</sup>. A relapse was counted if the mouse showed a reduction in score by at least one point for ≥2 days consecutively, followed by an increase of at least one point for ≥2 days consecutively. *P* values are provided for comparisons of mean relapse rates over 10 weeks by Mann-Whitney test, and number of mice with no relapses by Fisher's exact test. Animal experiments were conducted under approval from the Stanford University Institutional Animal Care and Use Committee.

**DNA tolerizing vaccines.** DNA constructs encoding PLP(139–151) and full-length mouse MBP, MOG and IL-4 in the pTARGET CMV promoter-driven mammalian expression vector (Promega) have been described<sup>10,14</sup>. cDNA encoding MAG and PLP were amplified from mouse brain cDNA (Clontech) using PCR and the following oligonucleotide primers: for MAG, 5'-CGCGCCGCCGCAAGATGATATTCCCTCGGCACC-3' and 5'-ACGGGATCCTCAGTGACAATCCCGGCTAGA-3'; for PLP, 5'-CGCGCGGCCGCGACATGGGC TTGTGTAGACTGT-3' and 5'-ACGGGATCCTCAGAACITGGTGCCTCGGC-3'. Amplified cDNAs were cloned into pTARGET. DNA plasmids were produced in the *Escherichia coli* strain JM-109 (Promega), purified using Qiagen Endo-Free Giga Prep kits (Qiagen) and their purity confirmed as described<sup>10</sup>. At day 7 or 8 after onset of and after partial recovery from acute EAE (day 17) induced with PLP(139–151), SJL/J mice were treated with weekly intramuscular injections divided between both quadriceps containing (i) 100 µg of pTARGET encoding IL-4, (ii) a mixture of 50 µg of pTARGET encoding IL-4 and 50 µg of pTARGET encoding PLP(139–151), (iii) the cocktail mixture containing 50 µg of each of four separate pTARGET plasmids encoding MBP, PLP, MOG and MAG or (iv) the cocktail mixture plus 50 µg of pTARGET encoding IL-4.

*Note: Supplementary information is available on the Nature Biotechnology website.*

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#### COMPETING INTERESTS STATEMENT

The authors declare competing financial interests (see the Nature Biotechnology website for details).

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